analysis of sterol dehydration products from refined rapeseed oil (the composition of which is of interest for detecting adulteration of other oils, e.g. olive oil). Sample preparation consisted of preparing a 1 : 5 dilution of the oil. The first LC column isolated the hydrocarbons (with the column backflushed by a stronger eluent after each analysis), while the second one separated the products of interest into groups, such that the closely related compounds could be separated by GC. The fractions from LC-LC and the related gas chromatograms are numbered.

Water Analysis

Brinkman, Vreuls, Noij and others have worked on the enrichment of organic materials from water on LC cartridges, followed by on-line liquid or thermal desorption into a gas chromatograph. The aim is a permanent, fully automated analysis of pesticides and other critical contaminants in rivers or the supply lines of water works. A standard procedure consists in extraction of 1–10 mL of water on short polymer-packed LC columns, which are then washed with clean water and dried by a stream of nitrogen. After desorption with ethyl acetate, the sample is transferred through the on-column or loop-type interface.

Conclusion

On-line LC-GC techniques are extremely powerful with regard to selectivity, sensitivity (as a result of the excellent clean-up) and efficiency (as most manual sample preparation is integrated into the automated analysis). It seems, however, that currently they are too demanding for widespread routine use.

See also: **III/Crude Oil: Liquid Chromatography. Terpenoids: Liquid Chromatography. Essential Oils:** Gas Chromatography. **Oils, Fats and Waxes: Supercritical Fluid Chromatography. Petroleum Products:** Liquid Chromatography. **Pesticides:** Gas Chromatography.

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Paper Chromatography

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Introduction

The techniques of paper chromatography and paper electrophoresis are sufficiently intertwined as to be worth considering together, as indeed they often were in books and reviews at the height of their popularity.

Paper Chromatography

The origins of paper chromatography have been traced back by some authorities as far as Pliny (23–79 AD), who described the use of papyrus impregnated with an extract of gall nuts to detect ferrous sulfate. Further examples of the use of paper chromatography can be seen in the 19th-century work of the German chemist Runge who described in his book *Zur Farbenchemie* the use of this type of separation for the investigation of inorganic mixtures. Subsequently another book (*Der Bildungstrieb der Stoffe*) by

Runge appeared, containing examples of this work. Further work in this area was undertaken by Schonbein and his student Goeppelshroeder, who investigated the technique of *Kapillaranalyse* (capillary analysis). However, these early studies seem to have stimulated little real interest and, although there appear to have been some limited further studies in the 1930s and 1940s, it was not until the seminal work of Consden, Gordon and Martin in 1944 on the analysis of amino acids in protein hydrolysates, and subsequent studies by Consden, Gorden, Martin and Synge, that paper chromatography made a major contribution to separations.

Paper chromatography is now obsolete, except perhaps as an inexpensive technique for teaching chromatography in schools and colleges. However the introduction of paper chromatography may truly be regarded as revolutionary, and was one of the innovations in partition chromatography that led ultimately to the award of the Nobel prize to Martin and Synge in 1952. One author stated, in a handbook on the topic, that 'By this stroke of genius, they changed the analysis of protein composition from a lifetimes' work to a 2–3-day simple technique that could be carried out in any laboratory'. So rapid was the adoption of the technique that a book on the subject published in 1954 contained nearly 4000 references to its use. Quotations from textbooks of the period contain statements such as 'Paper chromatography is so widely used that it is impossible to make more than a rough estimate of its application' or 'it can be stated that there is virtually no field of chemistry or biology in which paper chromatography has not made a substantial contribution to the furtherance of knowledge and understanding'.

However, despite its huge impact at the time, paper chromatography suffered from a range of problems that led to its rapid replacement by thin-layer chromatography (TLC), to which it was inferior in almost every respect. In particular, separations on paper were often very slow (often up to 10 or 20 h), and spots tended to be much more diffuse than, for example, separations on cellulose TLC plates.

The Practice of Paper Chromatography

Equipment

Probably the major advantage of paper chromatography, and one that ensured its rapid adoption, is the simplicity of the equipment required in order to perform it. Essentially this equipment is the same as that now used for TLC and all that is required is a suitable type of paper to act as the stationary phase, a means of applying the sample, a developing tank and a solvent system. A typical set-up for descending paper chromatography is illustrated in **Figure 1** showing, in addition to the tank and solvent reservoir the anti-siphon rod used to prevent excessive solvent flow from flooding the paper. Tanks were normally operated with the atmosphere saturated with the vapours of the solvent used for development in order to ensure good and reproducible results.

Solvents

In general, the solvent systems used in paper chromatography were based on mixtures of one or more organic solvents with water. Acids (HCl, acetic, etc.) or bases (aqueous ammonia) were added to control the ionization of the analytes. Typical solvent mixtures for amino acids, for example, might be composed of butan-1-ol, acetic acid and water; butan 1-ol, pyridine and water or phenol and water. For sugars, solvents based on ethyl acetate, pyridine and water; ethyl acetate, propan-1-ol and water or ethyl acetate, acetic acid and water were popular. For inorganic ions, solvent systems such as acetone, concentrated HCl and water; pyridine and water or butan-1-ol and HCl mixtures were suitable. In the case of some of these solvents the mixtures suggested separated on standing into two phases. In such circumstances it was customary to separate the two phases and use the aqueous layer to saturate the atmosphere in the developing tank and the organic layer as the eluent for chromatography.

Papers

The media used for both paper chromatography and paper electrophoresis were based on filter paper, with Whatman no. 1 being perhaps the most widely used and no. 3 also popular. However, paper manufactured by other companies was also used and Schleicher & Schull 2043b paper was popular according to some sources. Paper suitable for use in paper chromatography was also manufactured by Munktell, Macherey Nagel, Eaton-Dikeman and D'Arches. Not surprisingly, the availability of a range of papers produced numerous studies comparing the relative merits of the different products, eliciting the somewhat jaundiced comment in one review of the subject that 'These workers are not always in agreement … and it is probable that the differences introduced by their individual experimental methods are of a greater magnitude than the differences between the various grades of paper'. Some papers were available in slow, standard and fast grades with the speed of development controlled by the coarseness of the cellulose fibres and the packing

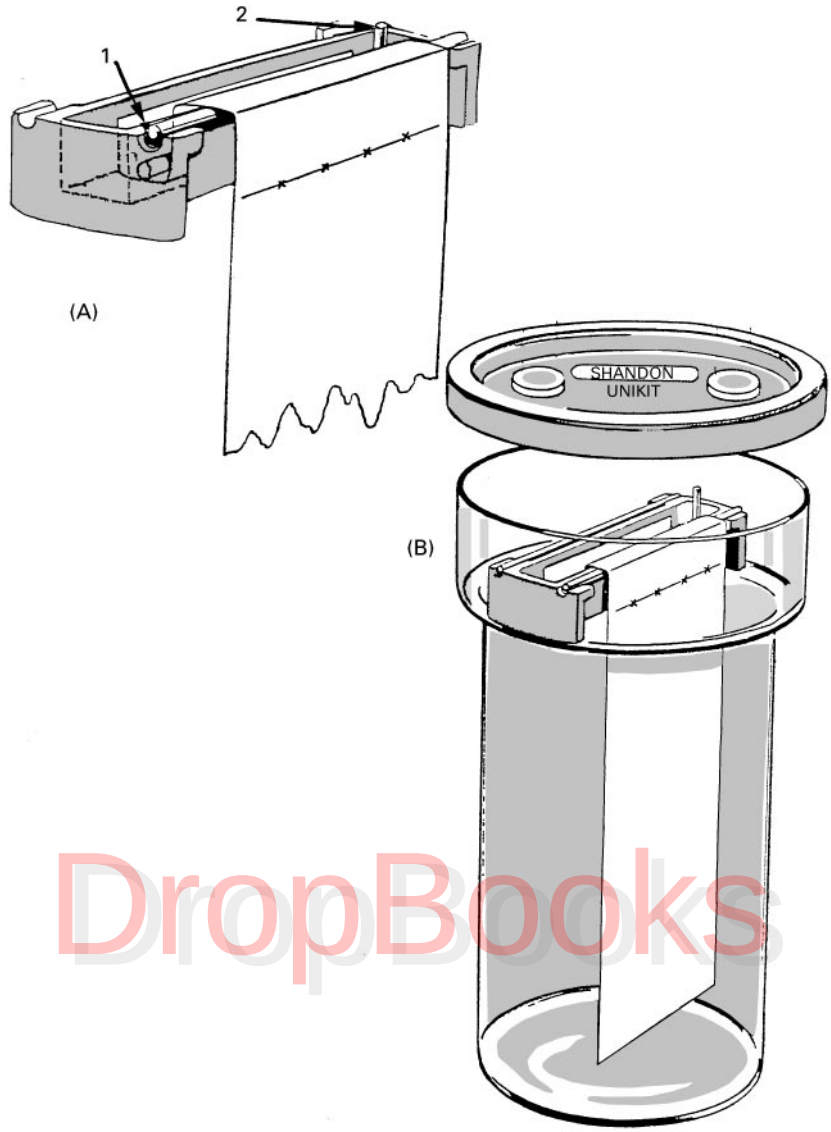

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Figure 1 A typical commercial system (Shandon Unikit) for descending chromatography. (A) Set-up for hanging the paper, which hangs freely over the anti-siphon rod (1) and dips into the solvent reservoir where it is held in place with the anchor rod (2). (B) The assembled reservoir and paper in place in the developing tank. At this point the solvent would be added and the tank closed with the lid.

density. In general, the standard papers gave the best compromise between speed and resolution, with fast papers more suitable for simple separations and the slow papers used where the greatest resolution was required. Whilst suitable for analytical work, these papers were often replaced in preparative applications by more specialized materials such as Whatman no. 3 MM and 31ET or Schleicher & Schull 2071. In addition to pure cellulose, a variety of modified papers were also produced, including ion exchange materials, acetylated or benzoylated papers, silicone oil-impregnated papers, as well as silica and alumina-impregnated papers.

Modes of Paper Chromatography

In most of its practical aspects (e.g. sample application, equipment such as developing tanks and visualization procedures), paper chromatography somewhat resembles TLC. The most noticeable difference is that, as the paper is not rigid, it must either be suspended from an appropriate support during development or arranged in such a way as to be self-supporting. Only the major types of paper chromatography are described below, but it should be noted that there were many minor variants of the technique (e.g. centrifugal, continuous).

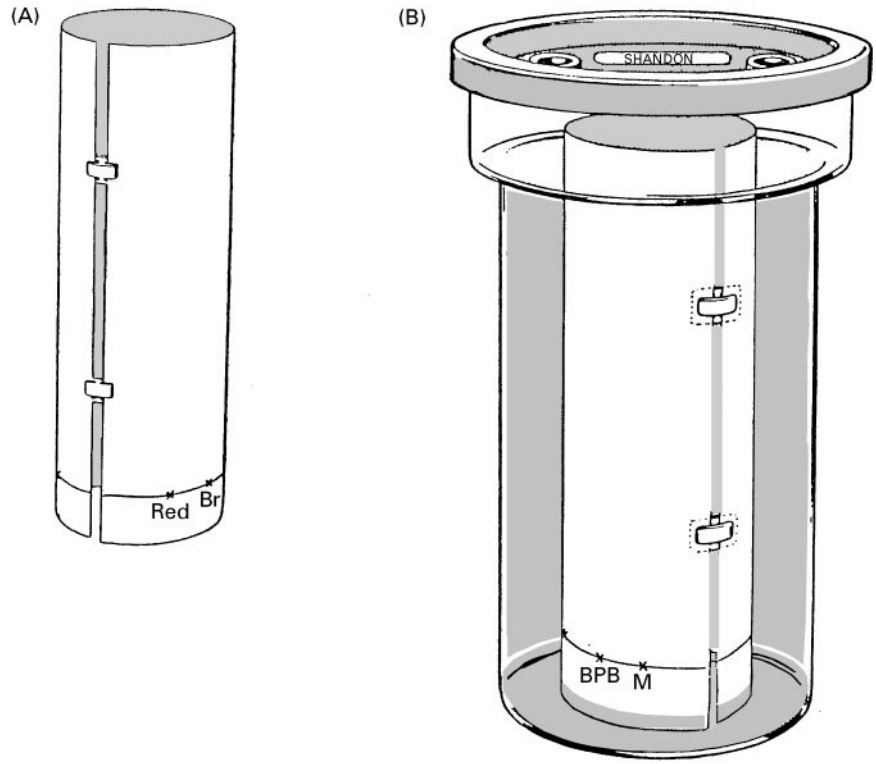

Figure 2 (A) One method of ascending chromatography involved the formation of a self-supporting cylinder, held together with tongued clips. The cylinder was then placed in a tank containing the solvent for development (B).

Ascending Paper Chromatography.

In the ascending mode of development the paper is suspended so that the lower edge is below the level of the solvent, and the solvent moves up via capillary action. An alternative to suspending the paper was to form a self-supporting cylinder from the paper. These arrangements are illustrated in **Figure 2**. As with TLC, multiple development, with either the same or different solvent, was used to improve resolution, although the time taken for each development must have made this especially tedious to perform.

Descending Paper Chromatography

The descending method of chromatogram development was that originally proposed by Martin and his co-workers. In descending paper chromatography the upper end of the paper is immersed in a solvent contained in a suspended trough so that the flow, initiated as in the ascending mode by capillary action, is sustained by gravity and will continue so long as there is solvent to feed it. This had the useful consequence that a sheet of any (practical) length could be used. In addition, the solvent could be allowed to run off the end of the paper, thus extending the chromatographic run if needed to improve resolution, or enabling compounds to be eluted from the paper and collected for further experiments. The results obtained for a particular sample/solvent system combination run in either ascending or descending mode were usually similar; however, the latter was generally faster.

Two-dimensional Separations on Paper

Where separations were not achieved in a single development, it was often possible to achieve the desired result using a second solvent system of different composition and development in a second dimension at 90° to the original direction of chromatography. Two-dimensional paper chromatography was first described by Consden, Gordon and Martin for the separation of 20 amino acids, but was subsequently widely employed. An additional possibility was the use of paper chromatography in one direction with paper electrophoresis (both high and low voltage) in the second. Indeed, there are numerous examples in the literature of either chromatography followed by electrophoresis or electrophoresis followed by chromatography. A typical example of the type of result that could be obtained using two-dimensional paper chromatography is shown in **Figure 3**, whilst **Figure 4** shows the combination of electrophoresis followed by chromatography in the second dimension for amino acids in fruit juice.

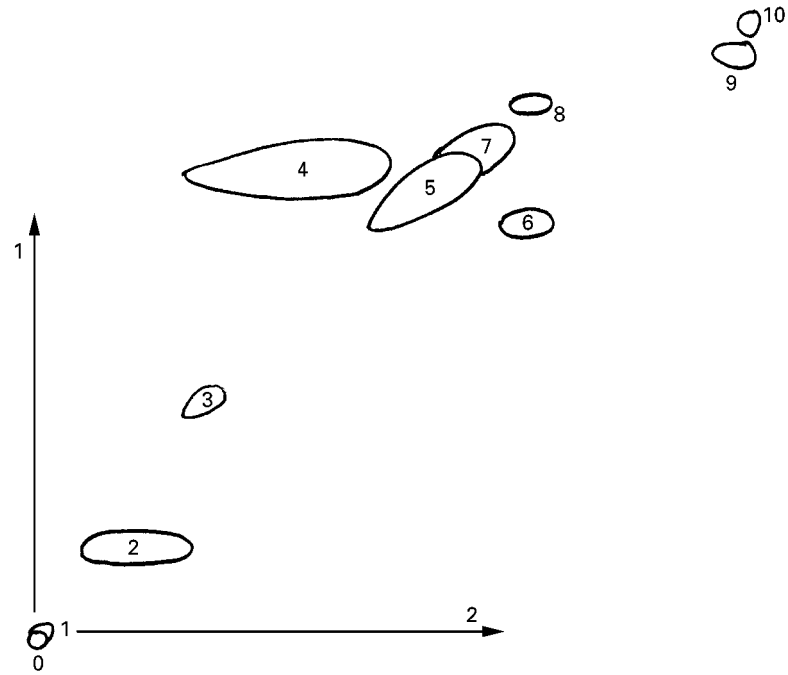

Figure 3 A two-dimensional separation of a mixture of black and brown ink using butan-1-ol–ethanol–2 mol L^{-1} aqueous ammonia (6 : 2 : 2) for the first development and butan-1-ol–acetic acid–water (6 : 1.5 : 2.5) for the second dimension, on Whatman no. 1 paper. Key: 0, origin; 1, dark blue material remaining at or near the origin; 2, yellow pigment; 3, pink pigment; 4, diffuse brown pigment; 5, pink pigment; 6, yellow pigment; 7, scarlet pigment; 8, pink pigment; 9 and 10, faint spots of orange and yellow pigments respectively.

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Horizontal or Circular Paper Chromatography

Horizontal (or circular) paper chromatography was performed in two ways. In the classical method, a spot of the sample to be analysed was placed at the centre of a circular filter paper. Then a short wick was made by making parallel incisions *c*. 2 mm apart from the edge of the filter paper to its centre. This wick was then cut to an appropriate size and bent so that it dipped into the solvent contained in a Petri dish. The general arrangement is shown in **Figure** 5A. Subsequently, apparatus became available that eliminated the need for cutting the paper to form a wick, and one such is shown in Figure 5B.

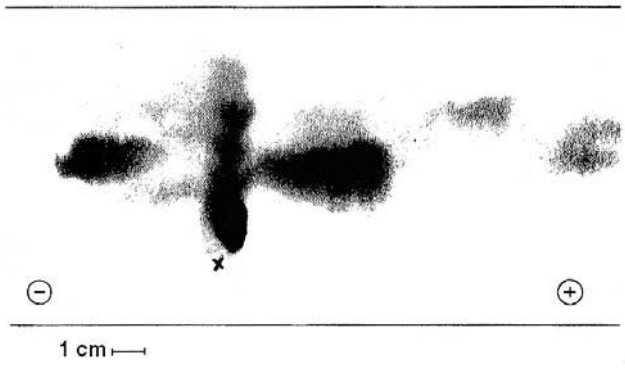

Figure 4 A typical two-dimensional separation of amino acids in orange juice effected by electrophoresis in pyridine–acetic acid followed by chromatography with butan-1-ol–acetic acid–water for the second. Detection with ninhydrin.

Preparative Paper Chromatography

For a time preparative paper chromatography was an important method for the isolation of substances, leading to comments such as: 'These methods are so well developed today that some laboratories prefer them to the methods of column chromatography'. The simplest methods of preparative paper chromatography were essentially scaled-up versions of the analytical methodology using either several sheets of paper or custom-made preparative cardboards (e.g. Schleicher & Schull 2071). In addition, techniques were developed such as the Chromatopile (a number of discs of filter paper in a tightly compressed stack to form a column, with development by either ascending or descending chromatography), the Chromatopack (strips or sheets of paper pressed together to form a block which was then developed as if it were a single sheet) or rolls of filter paper wound over a core of polyethylene and inserted into a polyethylene column (sometimes these rolls were placed in a pressurized jacket). Using such techniques the preparation of milligram quantities of material was readily achieved.

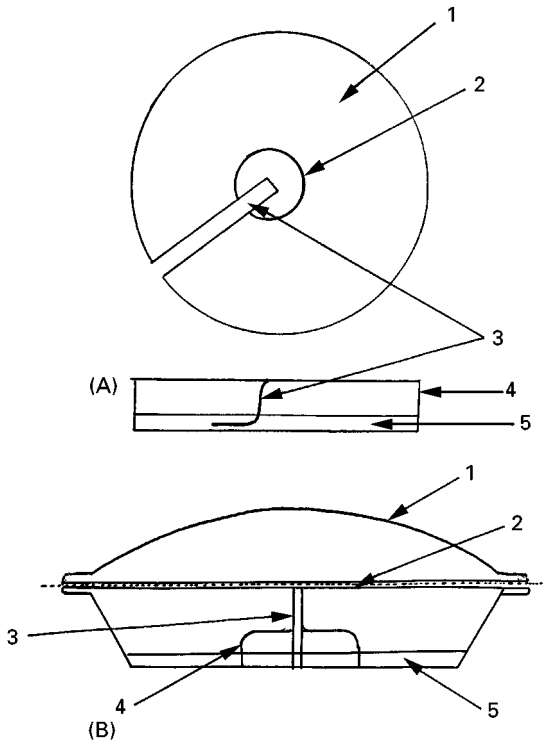

Figure 5 (A) Horizontal circular paper chromatography based on the method devised by Rutter. The upper part of this diagram shows 1, the paper; 2, the circle of sample applied to the paper (this could also be in the form of individual spots of different samples); and 3, the wick cut into the filter paper. The paper was supported on a Petri dish (4) containing the solvent (5) into which the wick was dipped in order to initiate development. Later the methodology was adapted by the introduction of a special development chamber which removed the need to cut a wick into the paper. One such, based on the apparatus devised by Kawerau, is shown in (B): 1, lid; 2, paper; 3, solvent capillary; 4, adjustable collar; 5, solvent.

Applications of Paper Chromatography

Given the importance of paper chromatography in its heyday, a list of its applications covers all types of analytes, including proteins, peptides, amino acids, poly-, oligo-, di- and monosaccharides, natural products, sterols, steroids, bile acids, pigment, dyes and inorganic species. A typical application to the separation of a series of inks is shown in Figure 6.

Paper Electrophoresis

It is possible to trace the development of paper electrophoresis back to the work of Konig, beginning in 1937 with a publication in Portugeuse. However, this work attracted little interest at the time and it was the later work of Wieland and Fischer on amino acids in 1948 and Durram on serum proteins (1949, 1950) that attracted the attention of the scientific community. Some measure of the importance of the technique in its heyday may be gained from the observation in a volume on paper chromatography and electrophoresis published in 1957 that 'more than 2000 papers dealing with the subject of zone electrophoresis have appeared. Over 90% of these have dealt with paper electrophoresis'. Faced with such apparent success, it is possible to forgive the enthusiasm of the authors of a subsequent manual, published in 1977, on the subject who felt able to say that: 'In fact, it can be truly said that the history of paper electrophoresis still lies before it'. In fact, as with paper chromatography, this type of electrophoresis is now considered by most workers to be entirely obsolete.

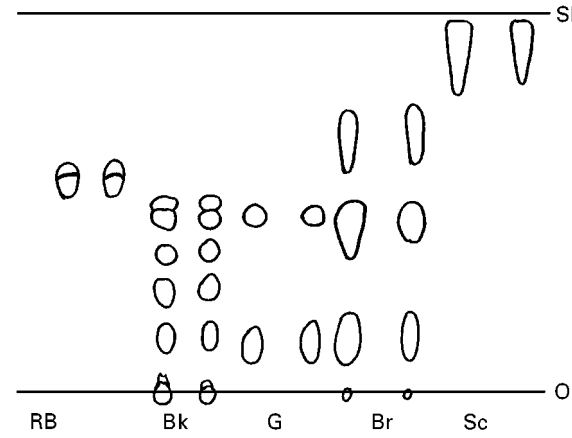

Figure 6 Separation, by ascending paper chromatography, of a series of ink samples (in duplicate). Key: RB, royal blue; Bk, black; G, green; Br, brown; Sc, scarlet; O, origin; SF, solvent front. Solvent system butan-1-ol–acetic acid–water (6 : 1.5 : 2.5) with Whatman no. 1 paper.

The Practice of Paper Electrophoresis

Paper electrophoresis can be broadly divided into three main techniques: low voltage, high voltage and continuous. Of these, the low voltage (up to 1000 V) technique was probably the most widely used.

Low Voltage Paper Electrophoresis

Strips of paper arranged either vertically or horizontally and moistened with the buffer were used. The application of the voltage (2–10 V cm^{-1}) used to perform separations generated some heat, but this was generally carried away by evaporation when the open strip method was used. In the open strip technique the paper was suspended between the

electrodes in the saturated gaseous phase of the developing chamber. This suspension was accomplished in a wide variety of ways, with one review of the technique stating that: 'Paper has been arranged in this chamber in almost every conceivable configuration, but it is usually either pulled horizontally taut or allowed to hang free from a central support at the apex'. Both the horizontal and hanging strip methods were reported to provide excellent resolution, but the latter was claimed to give better reproducibility. Other configurations included the semi-closed strip, where the paper was supported on one side by a cooled glass surface to enable temperature control, and the closed-strip method where the paper was either held between two glass plates or submerged in a nonpolar immiscible liquid (e.g. heptane or carbon tetrachloride). With the former system, evaporation was not permitted and pressure could be applied so as to control the amount of electrolyte taken up by the paper. Using the nonpolar immiscible liquid method, some heat was removed from the paper by convection and conduction to a thermostatic bath. A simple commercial low voltage paper electrophoresis apparatus, of the open strip type, is illustrated in **Figure 7**.

As well as one-dimensional seperations, two-dimensional paper electrophoresis was also performed when needed to improve particular separations.

High Voltage Paper Electrophoresis

The name high voltage electrophoresis was used to describe separations performed at voltages from 1 to 10 kV. The potential gradients used in high voltage systems were generally in the region of 50–100 V cm^{-1} and, as a consequence, one of the main problems encountered was heating. The apparatus used therefore required the presence of some form of heat exchanger to ensure that the heat was conducted away.

High voltage electrophoresis was considered to be best used for low molecular weight substances with many applications in amino acid analysis.

Continuous Electrophoresis

In continuous paper elecrophoresis the sample was applied continuously to the paper (**Figure 8**), enabling a considerable volume to be applied over time, allowing preparative separations to be performed. The layout of the paper in this type of separation is shown in the diagram. Thus, the paper is suspended vertically (often referred to as a curtain) so that the buffer solution flowed downwards (as in descending chromatography). A field was then applied across the direction of the flow, causing the ionic substances to be separated, as indicated in the figure. The individual components of the mixture could be collected into appropriate receptacles as they eluted from the paper.

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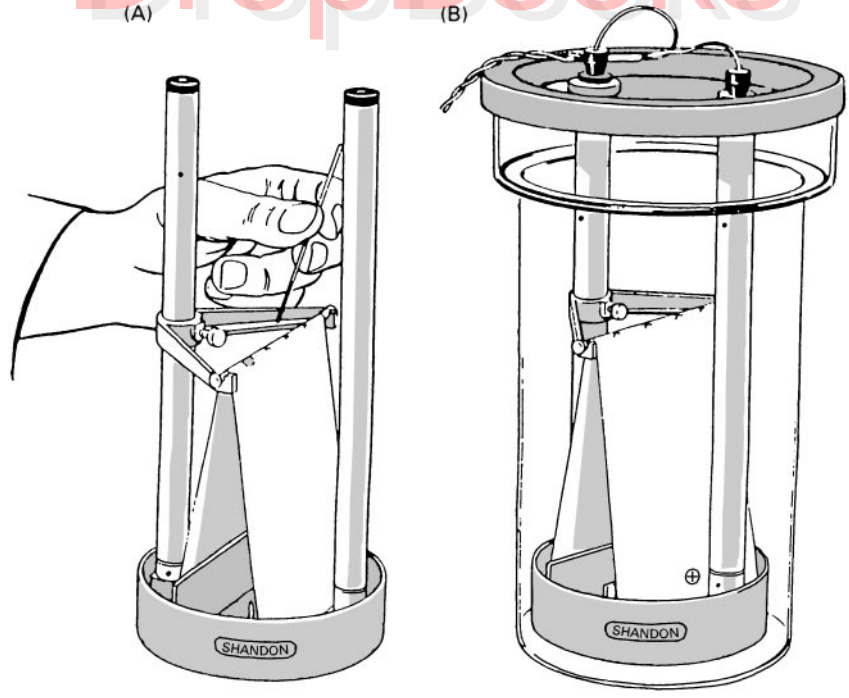

Figure 7 A simple commercial apparatus (Shandon Unikit) for paper electrophoresis. (A) The electrophoresis assembly showing the application of the samples to the paper which is suspended in a V shape via a glass rod. (B) Once prepared, the assembly is placed in the tank and the current switched on.

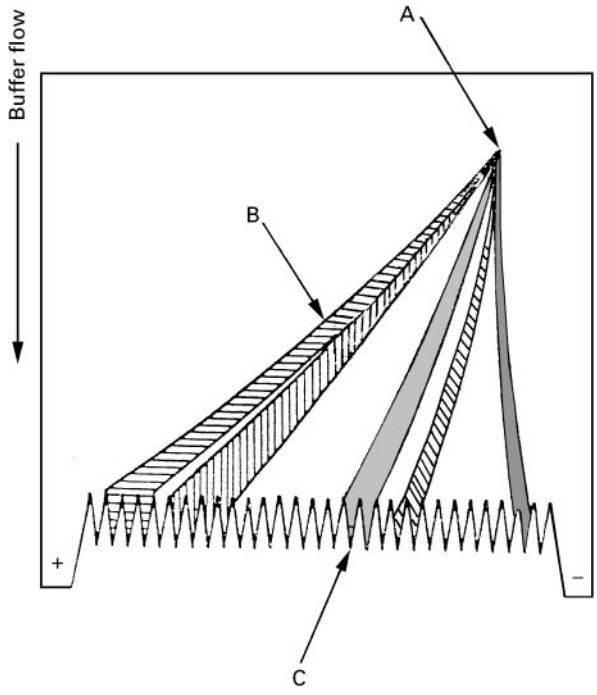

Figure 8 The arrangement for continuous electrophoresis. (A) Sample was applied continuously at this point and travelled down the paper under the influence of the flow of buffer. Current was applied at the point indicated by + and −, causing the components (e.g. B) to separate. They could then be collected into suitable receptacles as they eluted from the paper at C.

Applications of Paper Electrophoresis

As with paper chromatography, the applications of paper electrophoresis were legion and included amino acids, organic acids, natural products such as alkaloids, polysaccharides, nucleotides, proteins, peptides, pigments and inorganic species. The scope of the applications of paper electrophoresis is best appreciated by reference to the texts indicated in the section on Further Reading.

Detection and Quantification of Substances on Paper Chromatograms or Electropherograms

As in TLC, following separation the papers are removed from the developing chamber and dried. Coloured spots were visualized directly without difficulty, whilst those that fluoresce under UV irradiation were also detected relatively easily. In the case of colourless compounds, many of the visualization procedures, of varying degrees of specificity, currently used for this purpose in TLC were also used for detection after paper chromatographic separation, using either spraying or dipping. Although considered primitive by comparison with modern methods, separations on paper were also used for quantitative assays in addition to qualitative work. As with other planar methods, varying degrees of sophistication were employed, from comparison of the size of spots compared to a standard, cutting out the bands/spots and eluting the analytes for subsequent spectroscopic determination (i.e. UV, visible or fluorescence measurements) all the way up to the use of densitometry (with accuracies of $\pm 5\%$).

Conclusions

Paper chromatography and electrophoresis were once techniques of considerable importance but this is no longer the case. Whilst still useful as an aid to teaching chromatography in schools and colleges, there are virtually no situations where separations originally developed for paper chromatographic methods cannot now be performed faster and better by TLC. The same comments apply to the relationship between paper electrophoresis and the modern slab gel technique.

Acknowledgement

Figures 1, 2, 4 and 7 are adapted from a Shandon Southern Product Manual and are reproduced with permission.

See also: **I/Electrophoresis. II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Historical Development; Spray Reagents.

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